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CD105 (Endoglin): A Potential Anticancer Therapeutic Inhibits Mitogenesis and Map Kinase Pathway Activation

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Abstract. *Background:* CD105 is highly expressed on human activated endothelial cells (ECs), is an important component of the TGF- β 1 receptor complex and is essential for angiogenesis. CD105 expression is up-regulated in activated ECs and is an important potential marker for cancer prognosis. *Materials and Methods:* In vitro rat myoblasts transfected with the L-CD105 and S-CD105 transfectants. The transfectants were treated with TGF- β 1 for the angiogenesis study. *Results:* L-CD105 affects cell proliferation in the presence and absence of TGF- β 1, and inhibits p-ERK1/2, p-MEK1/2 and p-c-Jun in L-CD105 transfectants compared to controls. The induction of phospho-ERK1/2 following treatment with TGF- β 1 remained significantly lower in L-CD105 transfectants compared to controls. *Conclusion:* L-CD105 inhibits the phosphorylation of ERK1/2, MEK1/2, c-Jun1/2/3, and associated signalling intermediates. CD105 modulates cell growth and TGF- β 1 induced cell signalling through ERK-c-Jun expression.

CD105 (Endoglin) is a 180-kDa homodimeric integral transmembrane glycoprotein composed of disulphide-linked 90-95 kDa subunits and is a receptor component for TGF- β 1 and TGF- β 3 (1, 2). It is primarily found on activated angiogenic endothelial cells (ECs) of microvessels of cancer and numerous other angiogenic diseases (3-8). CD105 regulates a wide range of cellular and physiological

responses, including embryonic development, homeostasis, wound healing, chemotaxis, cell proliferation, differentiation, adhesion, migration, and apoptosis (4, 9-11). Overexpression of CD105 modulates TGF- β signalling by interacting with TGF- β receptor I (TGF- β RI) and/or TGF- β receptor II (TGF- β RII) on CD105 transfected L6E9 cells and human vascular smooth muscle cells through mitogen-activated protein kinases (MAPKs) (12, 13) and small-mothers-against-decapentaplegic (Smad) proteins (14, 15). Since CD105 overexpression was previously shown to be a marker of poor cancer outcome (16), and the phosphorylated ERK is a potential predictor of sensitivity to the treatment of carcinoma, as shown by various *in vitro* studies (17, 18), thus CD105 could be used as a potential therapy in cancer. Currently, CD105 is under phase 1 and phase 2 clinical trial in cancer patients (our and Seon *et al.*'s unpublished data).

CD105 has two isoforms, long-form L-CD105 and short-form S-CD105 (19), both of which bind to TGF- β 1 and TGF- β 3. These two isoforms have contrasting roles in angiogenesis with L-CD105 being pro-angiogenic and S-CD105 inhibiting angiogenesis (20). Since L-CD105 is expressed at a markedly higher level than S-CD105 in ECs, the overall balance tends towards a pro-angiogenic phenotype *in vivo* (21).

The expression of CD105 on ECs and other cells is up-regulated by both TGF- β and hypoxia (22), but their additive effect is substantially greater than by themselves (23). Vascular lesions (telangiectasia and arteriovenous malformations) in CD105 haploinsufficient hereditary hemorrhagic telangiectasia type I (HHT1) patients are associated with a loss of the capillary network (1, 24). The association of mutations of the CD105 gene with HHT1 indicates the importance of CD105 for normal vascular architecture. Knockout mice for CD105 die *in utero* due to defective angiogenesis (1, 25).

Our previous and other studies have shown that CD105 is a critical marker for the quantification of microvessel density staining in numerous types of human tumours (3, 26-31) and

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Key Words: CD105, MAPK, cell signalling, angiogenesis, TGF- β 1.

Table I. List of selected proteins identified by Kinexus phospho-protein array.

Protein name	Abbreviation epitope	L6M	L6L	L6S	T-L6M	T-L6L	T-L6S
Cyclin-dependent protein-serine kinase 1/2	CDK1/2 Y15	100%	−16%	18%	164%	54%	85%
Extracellular regulated protein-serine kinase 1 (p44 MAP kinase)	ERK1 T202+Y204	100%	−89%	−70%	133%	−18%	24%
Extracellular regulated protein-serine kinase 2 (p42 MAP kinase)	ERK2 T185+Y187	100%	−70%	11%	123%	−52%	57%
c-Jun proto-oncogene-encoded AP1 transcription factor	c-Jun S73	100%	−88%	−67%	148%	−44%	−37%
MAPK/ERK protein-serine kinase 1/2 (MKK1/2)	MEK1/2 S217/S221	100%	−55%	−81%	135%	−5%	−46%
MAP kinase protein-serine kinase 3/6 (MKK3/6)	MEK3/6 S189/S207	100%	−63%	−53%	134%	−31%	−21%
Protein-serine kinase C α	PKC α	100%	254%	133%	32%	259%	283%
Raf1 proto-oncogene-encoded protein-serine kinase (S259)	Raf1 (60)	100%	13%	159%	66%	144%	236%
Src proto-oncogene-encoded protein-tyrosine kinase (Y529)	Src (46)	100%	7%	17%	131%	169%	172%

T: TGF- β 1; L6M: Mock transfectants; L6L: L-CD105 transfectants; L6S: S-CD105 transfectants; T-L6M: TGF- β 1 treated mock transfectants; T-L6L: TGF- β 1 treated L-CD105 transfectants; T-L6S: TGF- β 1 treated S-CD105 transfectants. The results from Kinexus protein array were shown and the proteins were normalised as count per minute (CPM). The changes of 25% were as significant.

is involved in TGF- β 1 induced cell signal transduction (1, 32-35). Although, the intracellular mechanisms by which and how CD105 modulates TGF- β 1 signalling have been extensively studied, they need further clarification (7, 9, 36-46).

The roles played by CD105 in the tumour angiogenesis and the development had been widely studied (47-51). Previous studies have shown that CD105 was expressed in many types of tumours (22, 52-56).

The microenvironment of solid tumours consists of heterogeneous cell types such as endothelial (ECs), inflammatory (T cell and macrophages) mesenchymal stem (MSCs), fibroblasts, myofibroblasts (also called cancer-associated fibroblasts, CAFs), determines cancer prognosis (57-60). Most importantly apart from ECs, CD105 is also expressed on MSCs, CAFs and some subtypes of T-cells, is involved in their functional regulation and is a determinant of cancer development (45, 61). In an attempt to examine the role of CD105 on tumour angiogenesis and its effect on other cell types that are within the cancer microenvironment, we could not use human umbilical vein endothelial cells (HUVECs) or CAFs as a model system to overexpress CD105, because both these cell types express CD105 (3, 62, 63). In this study, we chose to use rat myoblasts stably transfected with human CD105 to determine the downstream signalling mechanisms of CD105. This may lead to designing anticancer approaches. We found that overexpression of L-CD105 in rat myoblasts induced growth inhibition and reduced ERK phosphorylation. This finding is consistent with the previous publications that

CD105 expression in cancer cells inhibits their growth and metastasis (64-67).

Materials and Methods

Stable human CD105 transfection in rat myoblasts. Preparation of stable rat myoblast CD105 transfectants expressing human L-CD105, S-CD105 and control mock transfectants was as described (68). No significant differences were observed between parental and mock transfectants in biochemical, functional and other studies (32).

Cell culture of human CD105 transfectants. The transfected rat myoblasts; mock (L6M), L-CD105 (L6L) and S-CD105 (L6S) transfectants were cultured in six-well plates (2×10^5 cells/well) as previously described (32). The mock (L6M), L- and S-CD105 transfectants (L6L, L6S) were initially seeded in six-well plates (2×10^5 cells/well) and utilised for western blotting as described previously (69).

Quantification of cell viability. Human CD105 transfected rat myoblasts were seeded and maintained in six-well plates at 1×10^5 cells/well (32). Total cell counts were undertaken at 24 h, 48 h and 72 h and following trypsinisation using a Coulter Counter (Beckman Coulter, High Wycombe, UK).

Immediately after the cell counting, their viability was ascertained using 0.04% trypan blue. Four hundred cells were counted for each condition at every time point and the number of dead cells was expressed as a percentage of the total cell population. There were no significant differences in cell viabilities amongst the three kinds of transfected rat myoblasts (data not included).

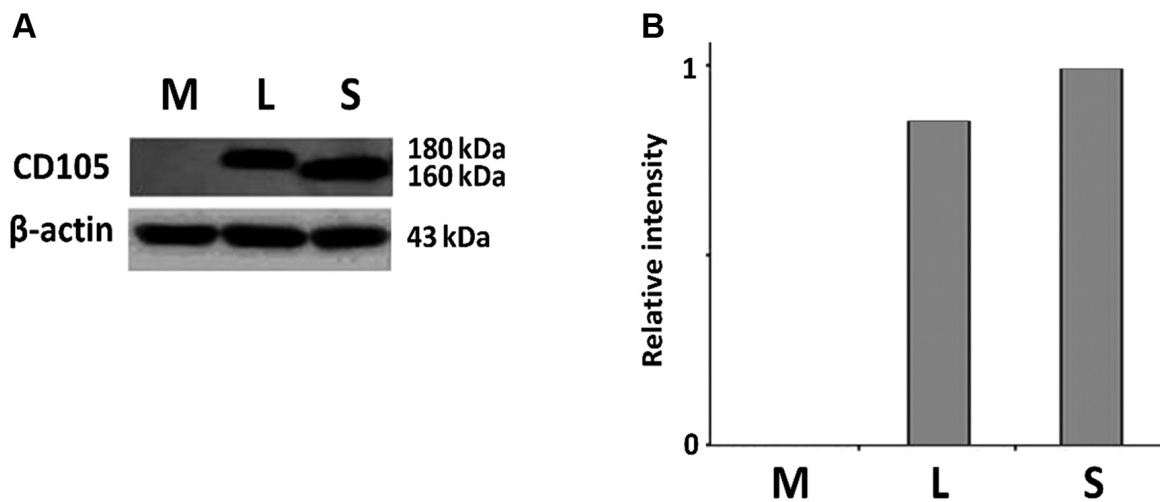


Figure 1. Western blotting showing that human CD105 were transfected into rat myoblasts successfully, whereas L-CD105 protein was overexpressed in L6L and S-CD105 protein was overexpressed L6S cells, but there was no CD105 expression in mock cells. β -actin was used as a loading control. The data are representative of two experiments of duplicate samples. M: Mock, L6M cells; L: L6L cells; S: L6S cells.

Cell proliferation following stimulation with TGF- β 1. Human CD105 transfectants were cultured in six-well plates at a density of 1×10^5 cells/well in 2 ml complete Dulbecco's Modified Eagle Medium (DMEM) containing TGF- β 1 (5 ng/ml, R&D Systems, Oxford, UK), in a humidified 5% CO₂/air atmosphere incubator at 37°C. After 48 h following trypsinisation, the cell numbers were counted using a Coulter Counter (Beckman Coulter, High Wycombe, UK).

Cell culture and protein extraction for western blot. L6M, L6L and L6S cells were seeded at 2×10^5 /well in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% antibiotics (10 U/ml penicillin and 100 μ g/ml streptomycin) in 6-well plate and incubated in a water-saturated, 5% CO₂ incubator at 37°C. For each experiment, the cells were starved in serum poor medium (cells were incubated in 1% FBS DMEM for 24 h) prior to the assay.

For the TGF- β 1 treatment study, the un-confluent starved cells were treated with TGF- β 1 (5 ng/ml, R&D Systems, Oxford, UK) for 5 min, 10 min and 30 min respectively. The cells were washed twice with ice-cold PBS and then were lysed by adding ice-cold Kinexus Lysis Buffer (Kinexus service, Vancouver, British Columbia, Canada) 300 μ l/well and incubated on ice for 1 min and then using a cell scraper to collect the cell lysates. After that, the cell lysates were transferred into a 1.5 ml Eppendorf tube and continued incubated on ice for 20 min. During the incubation, the cell lysates were sonicated every 2 min. Subsequently, the lysates were centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatants were collected, and aliquots stored at -80°C for later use.

Protein estimation and western blotting. Protein concentration was estimated using a BCA Protein Reagents (ThermoFisher Scientific, Cambridge, UK). The protein samples (30 μ g of each) were processed under reducing conditions by adding equal volumes of 2-fold concentrated sample buffer and then subjected to 10% SDS-PAGE (70).

Kinetworks™ Phospho-site protein Screen assay. Human CD105 transfected rat myoblasts were seeded (4×10^5 cells/dish) in 10 ml cell culture dishes (32). Sub-confluent cells were treated with TGF- β 1 (5 ng/ml) for 10 min and lysed with 1 ml of ice-cold cell lysis buffer (Kinexus, Vancouver, British Columbia, Canada). All cell lysates were sonicated twice (15 sec each time), and then incubated on ice for 20 min before ultracentrifugation for 30 min at $1 \times 10^5 g$ at 4°C. The resulting supernatant fractions were collected and assayed for protein concentration using a BCA Protein Reagents (ThermoFisher Scientific, Cambridge, UK) and adjusted to 1 mg/ml prior to use in the KPSS-1.3 protein array (Table I).

Statistical analysis. All data are presented as the mean \pm standard deviation from at least 3 independent experiments and were analysed using GraphPad Prism software version 7.0 for Windows (GraphPad Software, San Diego, CA, USA). The values were compared using a paired Student's *t*-test. The differences at $p < 0.05$ were considered statistically significant.

Results

Western blotting showed that human CD105 was expressed in both L6L and L6S cells but not in mock cells (Figure 1), wherein β -actin was used as a loading control. Figure 1 is representative of two separate experiments that gave similar results. All the experiments were repeated at least twice.

Cell growth studies demonstrated that in complete medium, L6M control cells grew significantly faster than L6L cells ($n=3$, $p < 0.05$; Figure 2) at 24 h, 48 h and 72 h. In contrast, L6S cell growth was not significantly ($p > 0.05$) different to control L6M cells on any of the time points.

Furthermore, L6M cells in the presence and absence of TGF- β 1 grew significantly faster than L6L cells over 72 h

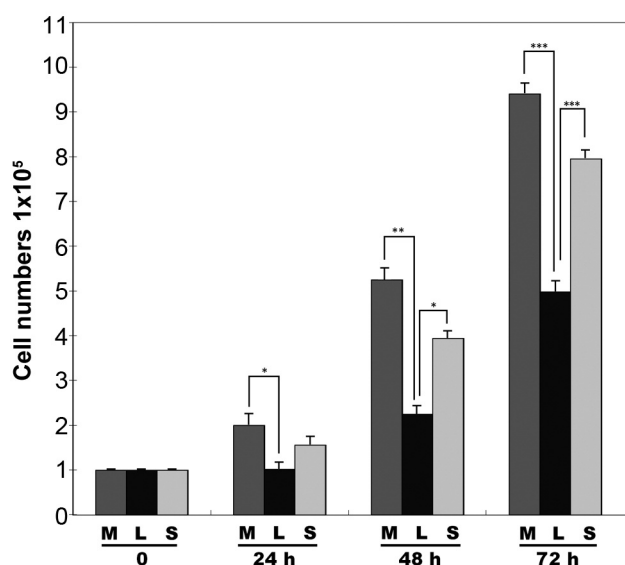


Figure 2. Cell proliferation assay showing a significant decrease in L6L after 24 h and 72 h of treatment ($n=3$; $p<0.05$). However, there was only a slight non-significant decrease in L6S cells, compared to L6M cells. The data represent results from three experiments, with samples measured in triplicate for each experiment. M: L6M cells; L: L6L cells; S: L6S cells.

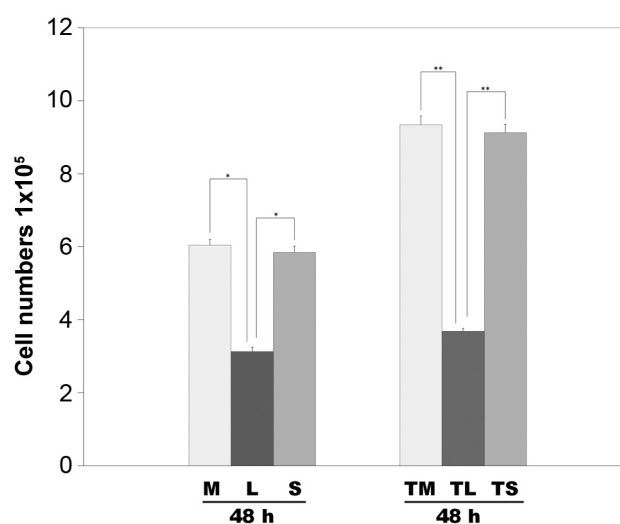


Figure 3. Cell proliferation assay displaying a significant inhibition in L6L cells after 72 h (significant, $n=3$, $p<0.05$) with and without TGF- β 1 treatment compared to control (mock cells without TGF- β 1 treatment). Data represent results from three experiments, with samples measured in triplicate for each experiment. M: L6M cells; L: L6L cells; S: L6S cells; TM: TGF- β 1 treated L6M cells; TL: TGF- β 1 treated L6L cells; TS: TGF- β 1 treated L6S cells.

($n=3$, $p<0.05$; Figure 3). The proliferation of L6L cells following TGF- β 1 treatment was almost completely nullified within 72 h ($n=3$, $p<0.05$) compared to control mock cells without TGF- β 1 stimulation (Figure 3). Figure 3 is representative of two separate experiments, utilising triplicate samples.

Cell signal transduction activation was analysed by a Kinexus phospho-protein (KPSS-1.3) array. De-regulated phosphorylated proteins were identified and showed a notable reduction of phospho-ERK1/2, phospho-MEK1/2, and phospho-c-Jun in L-CD105 transfectants (L6L) compared to mock cells (L6M). Whilst, all these proteins were less decreased in S-CD105 transfectants (L6S) compared to L-CD105 transfectants (L6L). Figure 4 shows phospho-ERK1/2, phospho-MEK1/2, and phospho-c-Jun expression were up-regulated after TGF- β 1 treatment in both L-CD105 and S-CD105 transfectants. The total proteins extracted from human CD105 transfected rat myoblasts in the Kinexus phospho-protein western screening (KPSS-1.3) array were detected by 10% SDS-PAGE, with β -actin used as a loading control. In all cases, data were obtained from duplicate spots in the Kinexus array.

ERK1/2 is implicated in cell signalling via the TGF- β 1 receptor. Western blotting results (Figure 5) showed the basal level of p-ERK1/2 in L6M cells was substantially higher than in both L6L and L6S cells. The p-ERK1/2 level was higher in L6S cells than in L6L cells. Figure 4 shows p-

ERK1/2 and p-MEK1/2 were significantly increased in both L6M and L6S cells. p-c-Jun1/2/3 was increased in TGF- β 1 treated L6M cells, but less so in TGF- β 1 treated L6L cells. p-c-Jun1/2/3 expression was at lower levels either in the presence or in the absence of TGF- β 1 in both L6L and L6S cells, but the L6L cells had decreased more than the L6S cells. In all cases, β -actin was used as a loading control and data represent results from two separate experiments of duplicate samples.

To examine ERK1/2 expression associated with TGF- β 1 signalling in transfected rat myoblasts, the cell lines were stimulated with TGF- β 1 at various times. Following treatment with human TGF- β 1, the ERK1/2 was activated within 10 min in mock (L6M) cells (Figure 5A). In TGF- β 1 treated L6L cells, p-ERK1/2 increased within 5 min and peaked at 10 min, although it remained significantly lower ($p<0.05$) than in mock cells. After peaking at 10 min, p-ERK1/2 declined gradually in L6L cells treated with TGF- β 1 but remained above basal levels (untreated L6L cells) after 8 h (data not shown). Similarly in TGF- β 1 treated L6S cells (where the basal levels were higher than in L6L cells but lower than in mock cells), p-ERK1/2 increased at 5 min, peaked at 10 min, and thereafter began to decline in a similar pattern to that observed in L-CD105 transfectants. The results showed that the relative increase in ERK1/2 activation was lower in L-CD105 transfectants compared with S-CD105 transfectants and mock cells.

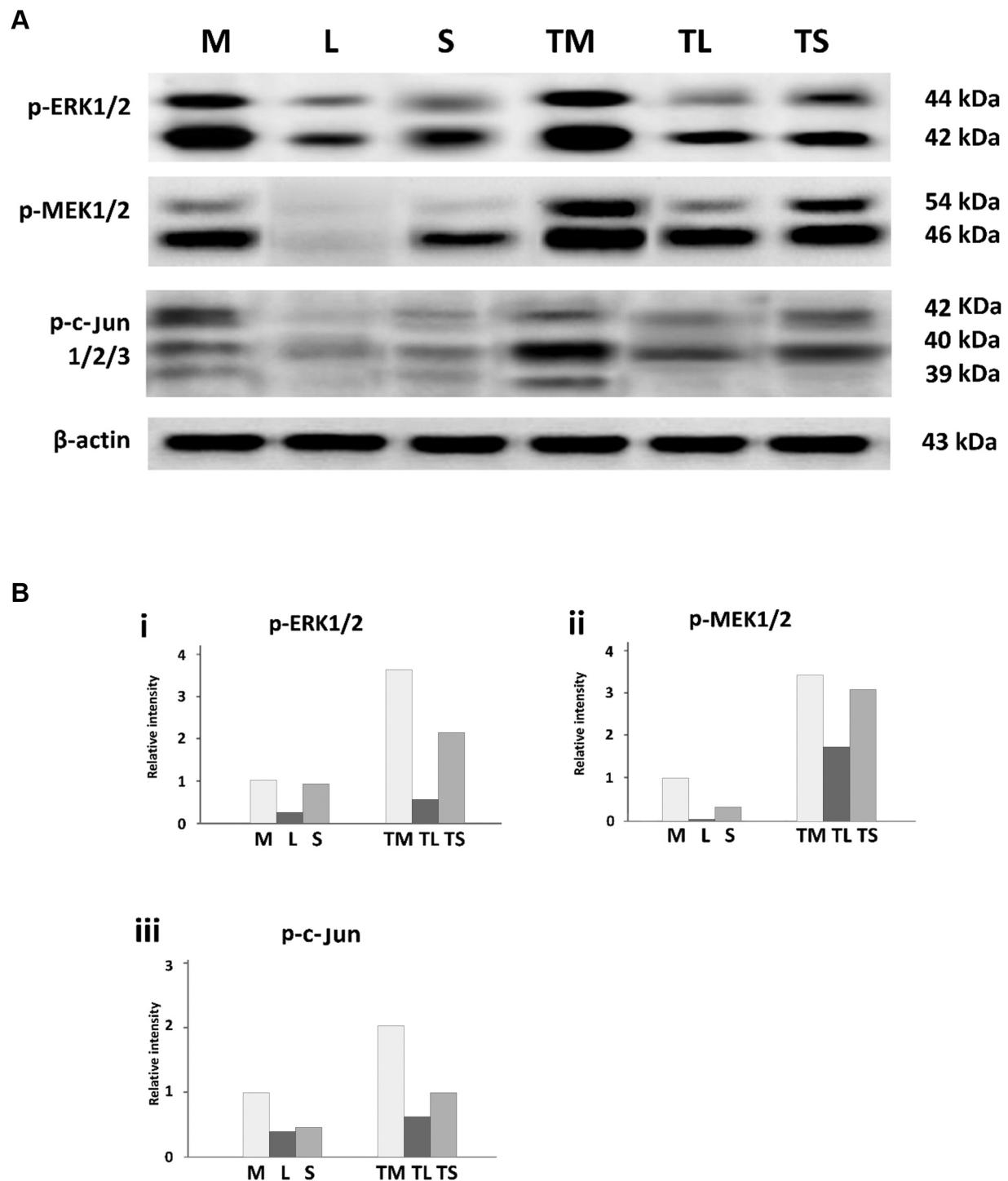


Figure 4. Proteomics study showing phosphorylated proteins in L6M, L6L, and L6S cells. Since CD105 appears to influence the activation of multiple signalling pathways, its effects on intracellular signal transduction in rat myoblast human CD105 transfectants were analysed using a Kinexus phospho-protein Western screening (KPSS-1.3) array. De-regulated phospho-proteins in both L-CD105 and S-CD105 transfectants were observed compared to control (mock transfectants). There was a significant reduction ($n=3$, $p<0.05$) in p-ERK1/2, p-MEK1/2, and p-c-Jun1/2/3 in L6L, compared to mock cells (control), whereas L6S cells were less affected. After TGF- β 1 treatment for 10 min, the p-ERK1/2, MEK1/2 and p-c-Jun were up-regulated in L6M and L6S but were less in L6L cells. The count per minute from Kinexus highlighted significant differences among the L6M, L6L, and L6S cells. The data represent a single experiment with proteins separated and analysed in duplicate. M: L6M cells; L: L6L cells; S: L6S cells; TM: TGF- β 1 treated L6M cells; TL: TGF- β 1 treated L6L cells; TS: TGF- β 1 treated L6S cells.

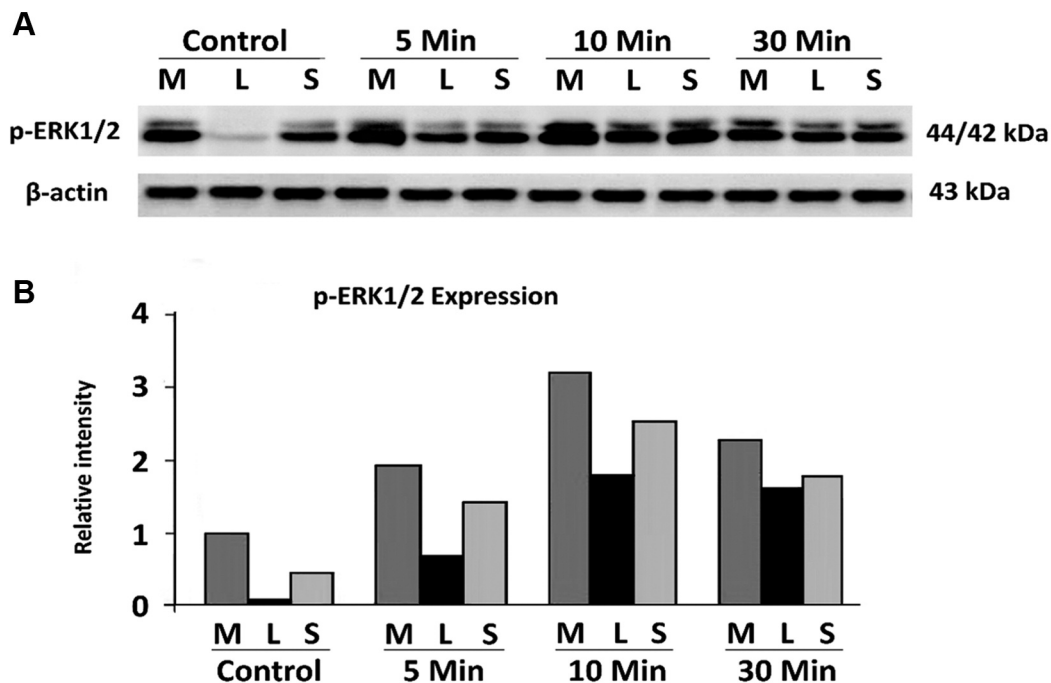


Figure 5. Western blot results demonstrates the phosphorylation of ERK1/2 (A) in L6L, L6S and L6M cells. The bar graph (B) suggests that the phosphorylation of ERK1/2 was notably reduced in L6L after TGF- β 1 treatment, while weaker inhibition was observed in L6S cells compared to L6M (control) cells. β -actin was used as a loading control. The basal levels of p-ERK1/2 in L6M cells was much higher than that in L6L cells. Following treatment with TGF- β 1, p-ERK1/2 in L6M cells increased after 5 min and peaked after 10 min, showing similar levels to those in L6S. In L6L cells, after TGF- β 1 treatment, p-ERK1/2 was also noticeably increased at 5 min, peaked at 10 min (but remained much lower than in L6M cells), and thereafter declined. L6S cells showed a steady increase after TGF- β 1 treatment. Data are representative of two experiments with duplicate samples in each experiment. M: Mock, L6M cells; L: L6L cells; S: L6S cells.

Discussion

CD105 is predominantly expressed in angiogenic vascular ECs in different types of cancers. It forms a complex with the TGF- β receptor and is able to modify TGF- β 1-mediated signalling pathways (1, 71). Like previous studies, Ilhan *et al.* concluded that overexpression of CD105 in colorectal cancers was associated with tumour angiogenesis and poor prognosis (8). However, the mechanism through which CD105 interacts with TGF- β 1 and how it interacts with intracellular signalling pathways is not fully elucidated.

An important finding emerging from this study is that mitogenesis/cell proliferation was reduced in both L- and S-CD105 transfectants compared to mock cells. Unexpectedly, L6L cells grew significantly more slowly than L6S cells, that contrasts with a previous publication (72). Further, TGF- β 1 induced proliferation of L-CD105 transfectants. We hypothesise that chronic overexpression of this receptor could lead to antagonistic or receptor feedback mechanisms which reduce the expression of phospho-ERK1/2 in these cells. In our phospho-protein study, ERK1/2, MEK1/2, and c-Jun1/2/3 were down-regulated in the L-CD105 but less so

in S-CD105 transfectants. As ERK is a key pro-mitogenic signalling intermediate, reduction in its expression/activation normally leads to reduced cell proliferation. This explains the fact that compared to L6M (mock transfectants), L6L cells grew more slowly than L6S cells.

This is the first study to show that both ERK, MEK and c-Jun phospho-protein expression is suppressed in rat myoblast CD105 transfectants. Specifically, western blot analysis revealed that phospho-ERK1/2 was significantly inhibited in both L-CD105 and S-CD105 transfectants. As previously reported, c-Jun plays an essential role in organogenesis during mouse embryonic development by regulating cell survival, apoptosis, and proliferation (73, 74). Therefore, it is possible that inhibition of the c-Jun pathway may influence proliferation in L-CD105 transfectants.

Although the phosphorylation of ERK remained inducible in CD105 transfected rat myoblasts after stimulation by TGF- β 1, the presence of the L-CD105 isoform significantly attenuated this response (12, 14). Chronic activation of the TGF- β 1 receptor by L-CD105 may have resulted in self-down-regulation or indeed induction in the activation of key signalling intermediates such as MEK, responsible for ERK

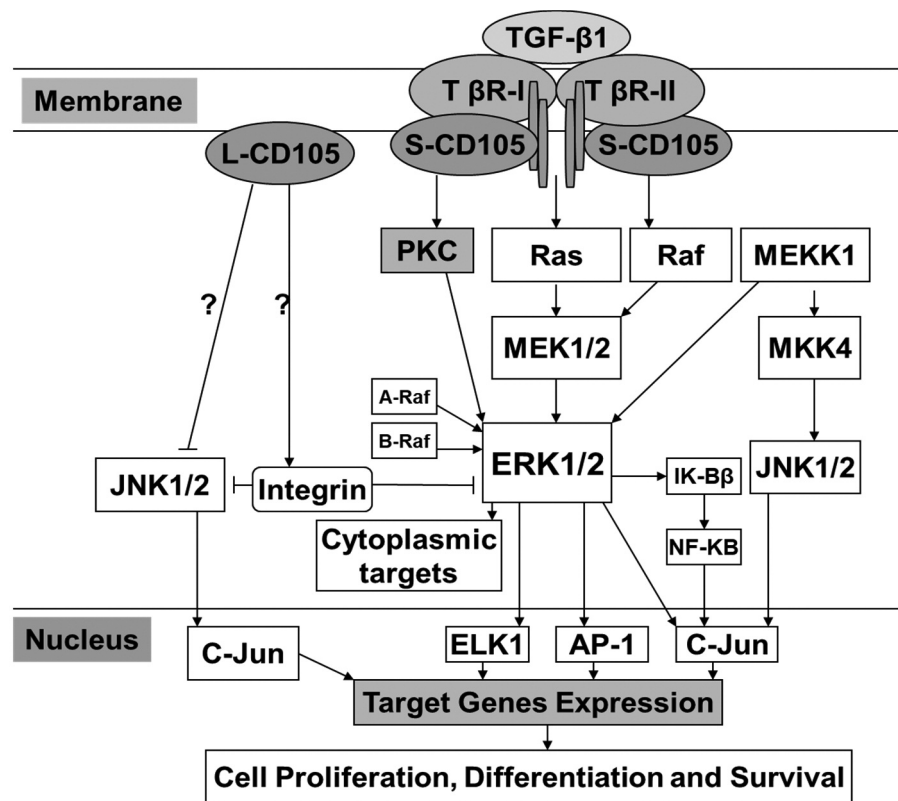


Figure 6. Schematic diagram representation of the TGF- β 1/ERK/c-Jun pathway regulation by the long and short form of CD105 that ultimately determines endothelial cell proliferation, differentiation and survival. TGF- β 1 stimulates ERK in the cell signalling pathway. Activation of the ERK pathway is most often associated with cell proliferation and cell survival. Well-characterized inhibitors of Raf-1 and MEK1/2 and the interaction of CD105 and TGF- β 1 in cell signalling pathways are highlighted. Chronic overexpression of L-CD105 may play a negative role in TGF- β 1 induced MAPK signalling pathway in human CD105 transfected rat myoblasts by a negative feedback mechanism or over-stimulation of ERK1/2 phosphorylation/activation [for more details see reference (78)].

phosphorylation. Taken together, these data suggest that over-expression of CD105 in rat myoblast transfectants may play an important role in the modulation of the ERK signalling pathway via TGF- β 1 signalling in these cells.

Previously we demonstrated that the phosphorylation of Smad3 was inhibited by CD105, which also inhibited TGF- β 1-Smad3 signalling. Overexpression of CD105 leads to phosphorylation of JNK, but there are no additive/synergistic effects on the JNK phosphorylation after TGF- β 1 treatment. And CD105 may either prohibit TGF- β 1/Smad3 signalling or inhibit the transcriptional activity of Smad3 up-regulating the phosphorylation of JNK (14). Here we found that over-expression of CD105 is also associated with the inhibition of p-ERK expression in rat myoblasts transfected with human CD105, and this might either interact with smad3 or act alone through ERK1/2. The stimulation of TGF- β 1 activates the ERK MAP kinase (69), but the exact role of ERK in the modulation of the TGF- β 1 signalling pathway is unclear (71, 75, 76). We hypothesise that the over-expression

of human L-CD105 in these cells modulates TGF- β 1 induced cell signalling and this occurs primarily through the ERK and c-Jun signalling pathways other than the smad3 pathway, but the detailed mechanisms need further exploration.

The decrease of p-ERK1/2 in L-CD105 transfectants may differently influence cell signalling, depending on which type of cell is under consideration. Although our data demonstrate that L-CD105 modulates TGF- β 1 induced cell signalling through a pathway involving ERK-c-Jun expression in human CD105 transfectants *in vitro*, further work is required to identify other participants in TGF- β 1 signalling pathway in these transfected cells. Similarly, the reduction in c-Jun expression could inhibit Smad 2/3 phosphorylation, and *vice versa*, the activation of c-Jun stimulated by TGF- β 1 can induce the phosphorylation of Smad 2/3 both *in vivo* and *in vitro* (76, 77).

In summary, we demonstrated that the phosphorylation of ERK is chronically decreased in the L-CD105 transfectants. The two isoforms of the human CD105 result in differences in gene

expression, in binding and activating the target genes and/or proteins, as well as in the regulation of cell mitogenesis (20).

In conclusion, CD105 modulates cell growth and TGF- β 1 induced cell signalling through a pathway involving ERK-c-Jun expression in human CD105 transfectants *in vitro* and that over-expression of L-form of CD105 may inhibit cell growth by modulating phosphorylation/activation of the ERK/c-Jun map kinase pathways (Figure 6).

Conflicts of Interest

All the Authors confirm that there are no conflicts of interest for this study.

Authors' Contributions

D Liu and J Ashworth drafted and edited the paper. D Liu carried out the cell culture, prepared the samples for the Kinexus study and western blot. K Ali performed proliferation assay, part of the western blot. A Fadel did the statistical analysis. B Guo contributed to the draft and figures. S Kumar and M Slevin designed the study and supervised the project.

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